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14. ABSTRACT

During the first year of the study, the PI has found that disrupting *Brca1* by either germline or epithelium-specific mutation in p18-deficient mice activates epithelial-to-mesenchymal transition (EMT) and induces dedifferentiation of luminal stem cells (LSCs), which associate closely with expansion of basal and cancer stem cells and formation of basal-like tumors. Mechanistically, BRCA1 bound to the *TWIST* promoter, suppressing its activity and inhibiting EMT in mammary tumor cells. Our findings showed that BRCA1 suppressed TWIST and EMT, inhibited LSC dedifferentiation and repressed expansion of basal stem cells and basal-like tumors. Thus, our work offers the first genetic evidence that Brca1 directly suppresses EMT and LSC de-differentiation during breast tumorigenesis.

15. SUBJECT TERMS

Brca1 suppresses EMT and stem cell dedifferentiation

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1. Introduction

Mammary epithelia are mainly composed of luminal and basal cells whose expansion and maintenance in adult mice are ensured by luminal and basal stem cells, respectively(1-2). Accordingly breast cancer is divided into two major subtypes—luminal and basal-like tumors—that develop through distinct mechanisms and exhibit different responsiveness to treatment(3). Mutation of *BRCA1* is frequently associated with basal-like breast cancer(4-7). How Brca1 controls cell lineage commitment, maturation and transformation in the mammary gland and tumor development remain to be defined and are the focus of this application.

p18^{Ink4c} (*p18*), an inhibitor of CDK4/6 and activator of the RB pathway, expresses significantly lower in human breast cancers, and deletion of *p18* in mice stimulates mammary luminal stem cell proliferation and leads to luminal tumor development(8). *Brca1* mutation in mice causes premature senescence making it very difficult to determine the mechanism of *Brca1* in the suppression of mammary tumors. Taking advantage of p18 deficient mice that rescue the growth defects caused by mutation of *Brca1*(9), we are able to study the role of *Brca1* in controlling mammary cell fate and tumor development. We discovered that mutation of *Brca1* altered luminal cell fate, down-regulated the expression of luminal differentiation genes, up-regulated the expression of basal genes, and activated Twist and other epithelial-mesenchymal transition (EMT)-inducing transcription factors in p18 deficient luminal and tumor cells. Germline mutation of *Brca1* converts p18 deficient luminal type tumors into basal-like tumors with EMT features. Ectopic expression of WT BRCA1 in *BRCA1* mutant human basal-like cancer cells suppresses Twist and EMT and knockdown of BRCA1 sensitizes luminal cancer cells to induction of Twist and EMT in response to TGFβ.

Based on these findings, we hypothesize that Brca1 suppresses Twist and EMT to prevent luminal stem and tumor cells from aberrant basal and mesenchymal differentiation. Reduction or loss of Brca1 activates Twist and EMT, which allow LSCs to gain a multipotent capacity and cancer stem cells (CSC) to enhance self-renewal potential and lead to luminal-to-basal and luminal-to-mesenchymal cell transformation. We propose three specific aims to test this hypothesis: (1) to determine the role of BRCA1 in suppressing EMT and basal differentiation of mammary luminal cells, (2) to determine the function of BRCA1 in suppressing EMT of breast cancer stem cells, and (3) to determine the molecular mechanism of Brca1 in suppressing TWIST.

2. Keywords

BRCA1, *p18^{ink4c}*, EMT, Luminal stem cell, Basal-like tumor

3. Overall Project summary

(1) Germline mutation of *Brca1* transforms *p18$^{-/-}$* luminal tumors into basal-like tumors with induction of EMT

In our previous studies, we reported that deletion of p18 in mice stimulates mammary LSC proliferation and leads to spontaneous luminal tumor development(8), and that germline mutation of *Brca1* in p18-deficient mice blocks the expansion of LSCs and transforms luminal tumors into basal-like tumors(9). Prompted by the highly invasive heterogeneous mammary tumors developed in *p18$^{-/-}$;Brca1$^{+/-}$* mice with various degrees of whorls and clusters of spindle-shaped cells within these tumors – typical morphological characteristics of mesenchymal cells(9) – we looked at molecular markers associated with EMT. We found that the majority of the luminal tumors from *p18$^{-/-}$* mice highly expressed E-cadherin (Cdh1), an epithelial marker, whereas basal-like tumors from *p18$^{-/-}$;Brca1$^{+/-}$* mice expressed very weak and heterogeneous Cdh1. In contrast, most (77%, n=13) of *p18$^{-/-}$;Brca1$^{+/-}$* tumors that developed after one year of age were stained positive for mesenchymal markers including fibronectin (Fn), vimentin (Vim), and CD29, while only 11% (n=19) of *p18$^{-/-}$* tumors that developed at a similar age were positive for these markers (Fig. 1A-C, Table 1). This observation suggests that heterozygous germline mutation of *Brca1* activates EMT in mammary tumor progression.

Consistently, *p18$^{-/-}$;Brca1$^{+/-}$* tumor cells that were positive for Ck5 expressed very low levels of Cdh1 and the majority of Fn positive cells co-expressed Ck5 (Fig. 1B). These data suggest, at the least, that some Ck5+ basal-like tumor cells lost their epithelial characteristics and gained mesenchymal features. In further analysis of these tumors for the expression of CD29 , a basal and mesenchymal marker(10) demonstrated to be enriched in breast CSCs(11-12), we found that 69% (n=13) of *p18$^{-/-}$;Brca1$^{+/-}$* tumors expressed various degrees

of CD29 positive tumor cells from 2%-60% while only 11% (n=19) of *p18*$^{-/-}$ tumors were positive for CD29 in 2-3% of tumor cells (Fig. 1C, Table 1). These observations support the notion that EMT activation, as previously demonstrated(10, 13), results in cancer cells gaining stem cell properties. Primary *p18*$^{-/-}$;*Brca1*$^{+/-}$ tumor cells formed more and larger colonies in matrigel than *p18*$^{-/-}$ tumor cells (Fig. 1E) and Ck5/Ck8 double positive tumor cells were frequently detected in *p18*$^{-/-}$;*Brca1*$^{+/-}$ tumors but rarely in *p18*$^{-/-}$ tumors, 1.1% (67/6100) vs. 0.04% (2/5120) (Fig. 1F and (8-9)) which further suggests increased CSCs in *p18*$^{-/-}$;*Brca1*$^{+/-}$ tumors. Together, these results indicate that heterozygous germline mutation of *Brca1* induces EMT, increases CSCs, and transforms p18 null luminal tumors into basal-like tumors.

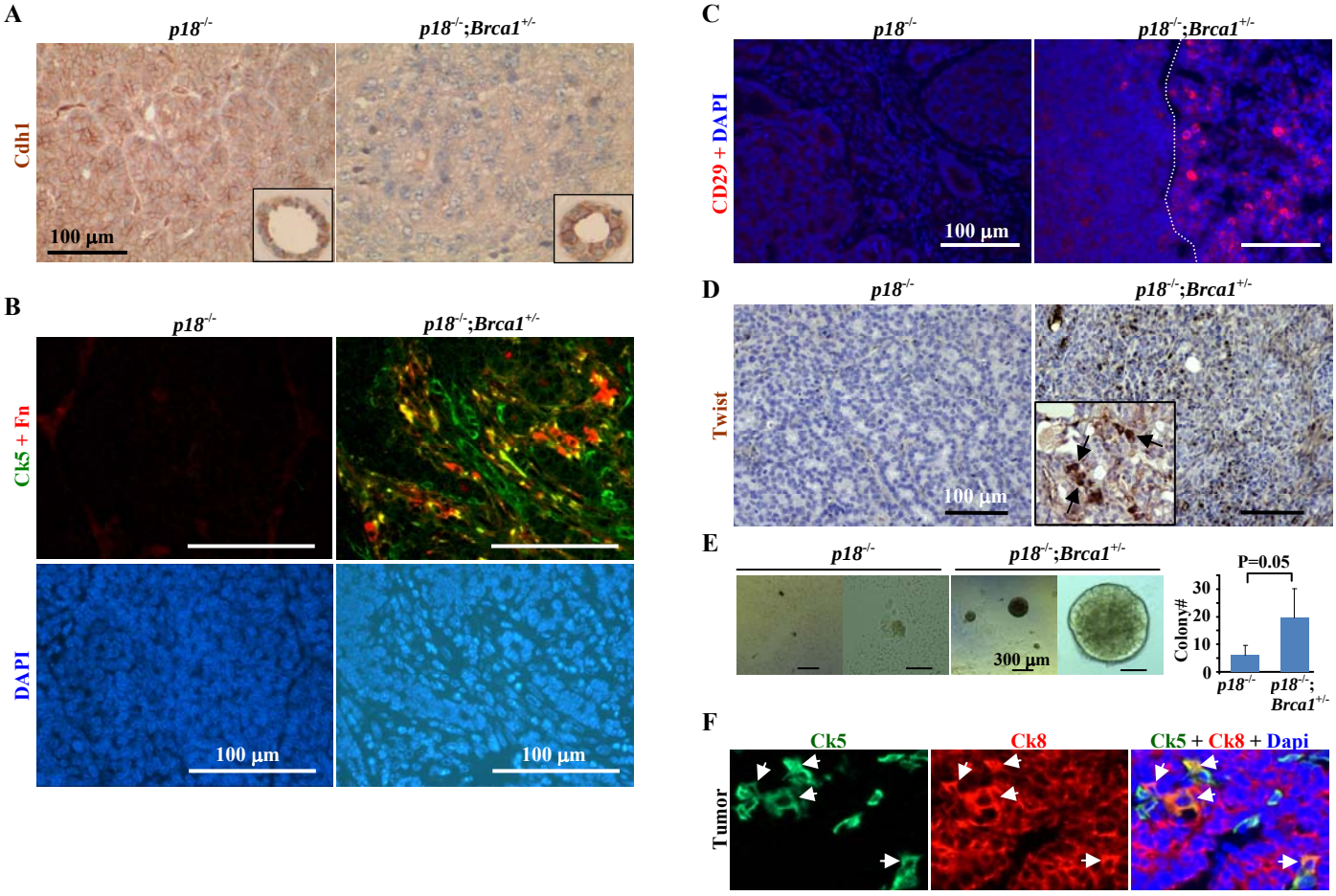

Figure 1. *Brca1* heterozygosity transforms p18-deficient luminal tumors into basal-like tumors with EMT features. (A-D) Representative immunostaining of tumors with Cdh1 (A), Ck5 and Fn1 (B), CD29 (C), and Twist (D). The inset in (A) shows staining of normal glands in the same mouse and in (D) shows staining of lung metastasis. (E) Tumor cells were cultured for two weeks and colonies larger than 30µm were counted. The bar graphs represent the mean ± SD of two tumors per genotype.
(F) Representative immunostaining of tumors from *p18*$^{-/-}$;*Brca1*$^{+/-}$ mice with Ck5 and Ck8. Ck5+Ck8+ cells are indicated.

(2) Germline mutation of *Brca1* activates EMT-TFs in mammary and tumor development

We then determined the expression of EMT-TFs and observed that 77% (n=13, >one year of age) of *p18*$^{-/-}$;*Brca1*$^{+/-}$ tumors were stained positive for Twist, Foxc1, Foxc2, Slug, and Snail in greater than 2% of cells per tumor whereas 16% (n=19, >one year of age) of *p18*$^{-/-}$ tumors were positive at similar ages (Fig. 1D, Table 1). Tumors with high expression of EMT-TFs showed high histological grade and strong invasive and metastatic potential as evidenced by EMT-TF positive staining in the invasive front of tumors and metastasized cancers (Fig. 1D). The expression pattern and percentage of positive cells in tumors stained for EMT-TFs and EMT markers were highly correlated with its genotype – *p18*$^{-/-}$ or *p18*$^{-/-}$;*Brca1*$^{+/-}$, – which not only confirms that germline mutation of *Brca1* promotes EMT in mammary tumors but that this induction of EMT is very likely a result of the aberrant activation of EMT-TFs in *Brca1* mutant tumors. We next isolated mammary epithelial cells (MECs) from tumor-free virgin mice and found that *Brca1*$^{+/-}$ and *p18*$^{-/-}$;*Brca1*$^{+/-}$ cells expressed

significantly less *Cdh1* and more EMT-TFs than WT or *p18*$^{-/-}$ cells (data not shown). These results indicate that EMT-TF activation in *Brca1* mutant MECs occurs prior to tumor initiation.

Tumor	Wt		*p18*$^{-/-}$		*Brca1*$^{+/-}$		*p18*$^{-/-}$;*Brca1*$^{+/-}$	
	<12 m	12-27 m	<12 m	12-22 m	<12 m	12-27 m	<12 m	12-22 m
Mammary Tumor	0/5	1/10[a] (10%)	4/16 (25%)	19/23[b] (83%)	0/3	1/11[c] (9%)	6/16 (38%)	13/15[d] (87%)
Metastasis[e]		0/1	0	1/19		0/1	0	4/13
ERα+ tumor **% ERα+ cells/tumor**		1/1 5%	3/4 2-40%	15/19 2-40%		0/1	1/6 <2%	2/13 <2%
Ck5+ tumor **%Ck5+ cells/tumor**		0/1	0/4	3/19[f] 1-5%		1/1 ~2%	4/6 2-20%	11/13[g] 2-95%
EMT marker+ tumor[h]		0/1	0/4	2/19 (11%)		0/1	2/6 (33%)	10/13 (77%)
EMT-TF+ tumor[i]		0/1	0/4	3/19 (16%)		1/1 (100%)	3/6 (50%)	10/13 (77%)

Table 1. Characterization of spontaneous mammary tumors derived from mutant mice
[a] 24-month-old tumor-bearing mouse.
[b] Most tumor-bearing mice were 12-16 months old and the oldest was 22 months old. One male developed mammary tumor.
[c] 25.5-month-old tumor-bearing mouse.
[d] Most tumor-bearing mice were 12-16 months old, and the oldest was 20 months old. One male developed mammary tumor.
[e] Mammary tumors metastasized mostly to the lung except one to a blood vessel in a *p18*$^{-/-}$;*Brca1*$^{+/-}$ mouse.
[f] One tumor stained positive for Ck5 in ~5% tumor cells and the other two were positive in ~1% tumor cells.
[g] Two tumors stained positive for Ck5 in ~95% tumor cells.
[h] At least two EMT markers (decreased Cdh1, increased Vim, Fn1, Sma or Cd29) were detected in >2% tumor cells.
[i] At least two EMT-inducing transcription factors (EMT-TFs), which include Twist, Slug, Snail, Foxc1 and Foxc2, stained positive in >2% tumor cells.

(3) Specific deletion of *Brca1* in mammary epithelia activates EMT and induces aberrant differentiation of LSCs

To directly test the function of Brca1 in controlling and transforming MECs as well as to determine the implications of loss of Brca1 on mammary tumorigenesis, we generated *Brca1*$^{f/f}$;MMTV-cre^{+} and *Brca1*$^{f/-}$;MMTV-cre^{+} mice with and without *p18* mutation in which MMTV-cre (MC) is active in virgin epithelia but not in stroma(14-15). Using these mice also enabled us to rule out the impact of *Brca1* mutant stroma on mammary stem cell self-renewal and tumorigenesis.

Brca1$^{f/-}$;MC and *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC breasts expressed <5% of Brca1 protein and mRNA relative to the levels in *Brca1*$^{f/+}$;MC and *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC, indicating an efficient and near complete depletion of Brca1 in the mammary epithelia (Fig. 2A, B). Similarly, *Brca1*$^{f/f}$;MC and *p18*$^{-/-}$;*Brca1*$^{f/f}$;MC breasts expressed <20% of Brca1 protein and mRNA relative to the levels in MC and *p18*$^{-/-}$;MC (data not shown). Consistent with the data from *Brca*$^{+/-}$ mice(9), the expression of *Gata3*, *Cdh1*, and *Epcam* – genes associated with luminal differentiation – in *Brca1*$^{f/-}$;MC and *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC breasts was significantly reduced relative to *Brca1*$^{f/+}$;MC and *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC breasts (Fig. 2A, B), suggesting that loss of Brca1 impairs luminal differentiation. MECs from *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC mice showed increased mammosphere-forming ability than those from *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC mice. Most *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC mammospheres were 35-45μm and none larger than 100μm whereas 10-15% of *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC mammospheres were larger than 100μm. The average *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC mammosphere was significantly larger than that of *p18*$^{-/-}$;*Br*$^{f/+}$;MC mammospheres (Fig. 2C). These results suggest that Brca1 deficiency increased the self-renewal capacity of *p18*$^{-/-}$ mammary stem cells. Accordingly, MECs from *p18*$^{-/-}$;*Brca1*$^{f/-}$;*MC* mice formed more colonies than those from *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC mice and *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC mammospheres expressed significantly higher levels of EMT-TFs than those of *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC (Fig. 2D, E). These results confirms that loss of Brca1 activates EMT-TFs, which is likely responsible for the induction of EMT and increased mammosphere- and colony-forming potential in *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC MECs.

We then performed FACS and found that *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC MECs had a reduced CD24^{+}CD29^{-} LSC-enriched population and increased CD24^{+}CD29^{+} BSC-enriched population compared to *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC MECs at 22 weeks of age (Fig. 2F). Similar, but less significant, trends were also observed in *p18*$^{-/-}$

;*Brca1*$^{f/+}$;MC mice relative to *p18*$^{-/-}$;MC mice at 16 weeks of age (data not shown). These results suggest that Brca1 deficiency results in the expansion of BSCs and blockage of LSCs, the latter of which is consistent with our findings derived from heterozygous germline *Brca1* mutant mice(9).

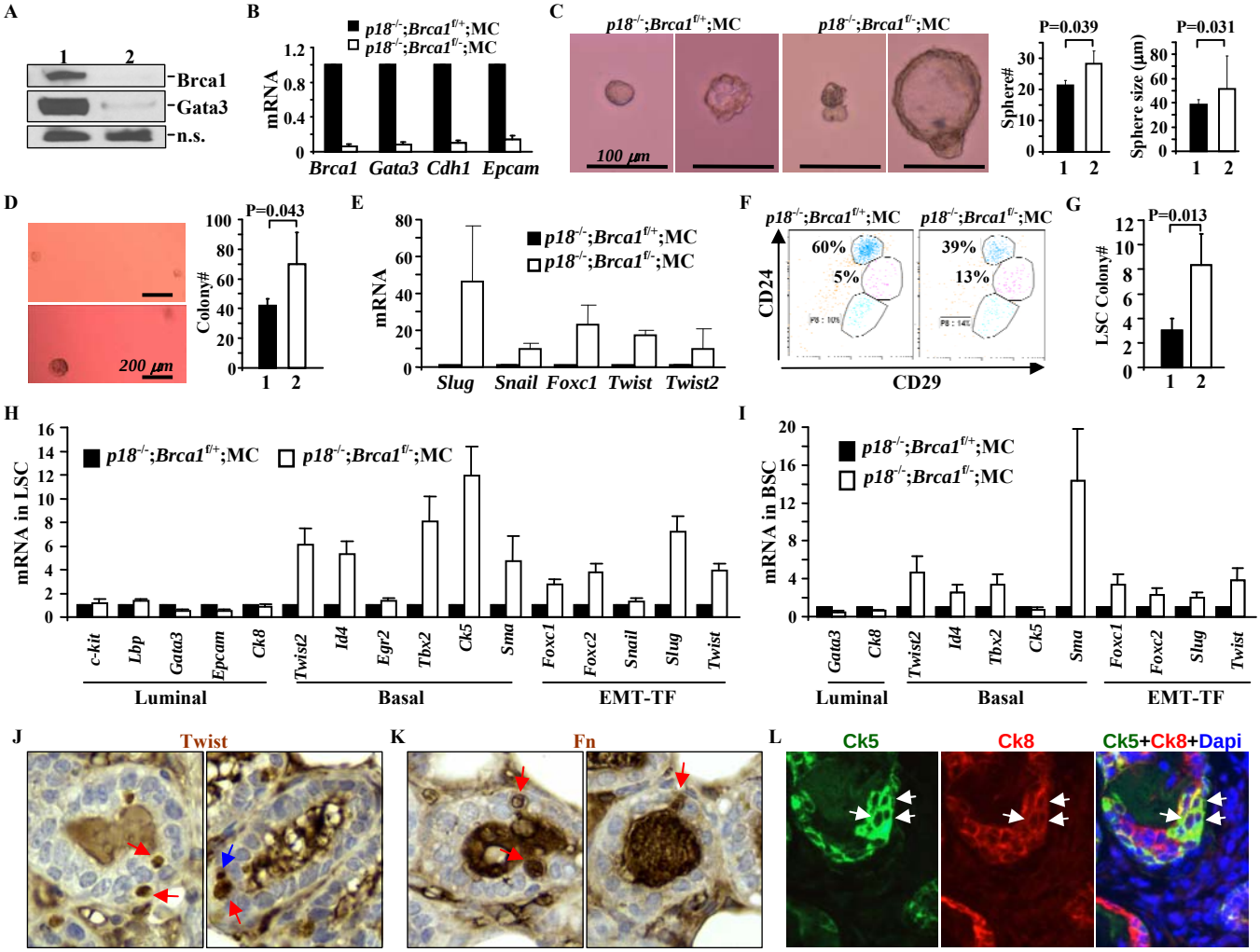

Figure 2. Deletion of Brca1 in mammary epithelia inhibits luminal differentiation and activates EMT-TFs in mammary stem cells. (A, B) Mammary tissues from *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC (Lane 1) and *p18*$^{-/-}$;*Brca*$^{f/-}$;MC (Lane 2) mice were analyzed by western blot (A), and Q-RT-PCR (B). n.s., non-specific band. Q-RT-PCR data are expressed as the mean ± SD from triplicates of each of three separate mice. (C, D) Mammary cells were analyzed by mammosphere (C) and colony formation assay (D). The number of spheres larger than 30μm, the sizes of spheres, and the number of colonies larger than 30μm were quantified.1, *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC; 2, *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC. The bar graphs represent the mean ± SD of two animals per genotype. (E) RNA from mammospheres was analyzed. Data are expressed as mean ± SD from triplicates of each of two separate mice. (F) Mammary cells were analyzed by flow cytometry. (G) FACS-sorted LSCs from (F) were analyzed by colony formation assay. The bar graphs represent the mean ± SD of two animals per genotype. 1, *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC; 2, *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC. (H, I) RNA from LSCs (H) and BSCs (I) was analyzed. Data are expressed as the mean ± SD from triplicates of each of two separate mice. (J, K, L) Tumor-free mammary glands from *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC mice were stained with antibodies against Twist (J), Fn (K), Ck5 and Ck8 (L). Twist or Fn positive ULLC (red arrows) and SLC (blue arrows), as well as Ck5+Ck8+ epithelial cells (white arrows) are indicated.

FACS-sorted cells of the BSC-enriched population expressed higher basal genes (*Twist2*, *Id4*, and *Tbx2*) and lower luminal genes (*c-kit*, *Epcam*, and *Gata3*) than those of the LSC-enriched population, confirming that these cell populations are, as reported(16), the basal and luminal cell enriched populations, respectively (data not shown). LSCs derived from *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC mice formed more colonies in matrigel and expressed lower luminal and epithelial genes and significantly higher basal genes and EMT-TFs when compared with *p18*;*Brca1*$^{f/+}$;MC LSCs (Fig. 2G, H). Consistently, LSCs from *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC mice also expressed lower luminal genes and higher basal genes and EMT-TFs than those from *p18*$^{-/-}$;MC mice (data not shown). These

results indicate that haploid or near complete loss of *Brca1* in mammary epithelium not only inhibits the expression of luminal genes but also stimulates the expression of basal genes and EMT-TFs in *p18*$^{-/-}$ LSCs. Interestingly, expression of basal genes and EMT-TFs was also significantly increased in the BSCs from *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC mice relative to those from *p18*;*Brca1*$^{f/+}$;MC mice (Fig. 2I). Together, these results suggest that Brca1 deficiency results in the expansion of BSCs that is likely, at least partially, resulting from the dedifferentiation of LSCs.

We have previously analyzed five histologically distinct epithelial cell populations and defined the small light cell (SLC) and undifferentiated large light cell (ULLC) populations as enriched for stem and luminal stem/progenitor cells, respectively(8). To determine the impact of EMT on stem/progenitor cell populations *in situ*, we examined tumor-free mammary glands and found that Twist or Fn positive MECs were frequently detected in *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC or *p18*$^{-/-}$;*Brca1*$^{f/f}$;MC mice but not in *p18*$^{-/-}$;MC mice and that most, if not all, Twist or Fn positive cells were either SLC or ULLC, ULLC in particular (Fig. 2J, K). Furthermore, Ck5 and Ck8 double positive epithelial cells were also frequently detected in *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC but not in *p18*$^{-/-}$;MC mammary (Fig. 2L, and data not shown). These results further suggest that loss of Brca1 in MECs activates Twist, induces EMT, and leads to dedifferentiation of LSCs.

(4) Specific deletion of Brca1 in mammary epithelia recapitulates basal-like tumorigenesis and EMT activation

To determine the tumorigenic impact of specific loss of Brca1 in mammary epithelia, we first examined *Brca1*$^{f/-}$;MC and *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC mice and found that no hyperplasia nor tumors developed in 5 female *Brca1*$^{f/-}$;MC mice at 10-12 months of age. Of the 8 *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC mice examined at similar ages, all developed mammary hyperplasia though no mammary tumors were detected. A majority (7/8) of *p18*$^{-/-}$;*Brca1*$^{f/-}$;MC mice died at early ages from carcinomas in the pancreas, skin, pituitary or lung (data not shown), very likely due to active MMTV-Cre expression and near complete deletion of Brca1 in these tissues(17), which prevented us from observing the relatively late onset mammary tumorigenesis in these mice. These results, however, confirm the previous findings that loss of Brca1 alone is insufficient to promote tumorigenesis and that Brca1 cooperates with p18 to control tumorigenesis.

We then examined *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC and *p18*$^{-/-}$;*Brca1*$^{f/f}$;MC mice and found that 1 of 4 *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC mice and 4 of 5 *p18*$^{-/-}$;*Brca1*$^{f/f}$;MC mice developed mammary tumors in 12-16 months (Fig. 3). In accordance with the tumors developed in *p18*$^{-/-}$;*Brca1*$^{+/-}$ mice, mammary tumors in *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC and *p18*$^{-/-}$;*Brca1*$^{f/f}$;MC mice were also highly heterogenous, poorly differentiated, and more aggressive than those developed in *p18*$^{-/-}$ mice (Fig. 3, Fig. 1, and data not shown). About 25-30% *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC tumor cells were spindle-shaped and were positive for Twist and Fn (Fig. 3A, B), and more than 40% of the tumor cells were positive for Ck5 and negative for Cdh1 or Ck8 (Fig. 3C, D), indications of a basal-like tumor undergoing EMT. The *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC mammary tumors also expressed 1/3 of *Brca1* and 1/5 of *Gata3* relative to the tumor-free mammary tissues of the same mouse (Fig. 3E), confirming deficient Brca1 and downregulation of Gata3 in the tumor.

More than 25% of tumor cells were spindle-shaped in all four *p18*$^{-/-}$;*Brca1*$^{f/f}$;MC mammary tumors and two displayed more than 90% spindle-shaped cells (Fig. 3F). These tumors were also positive for Twist and Fn (Fig. 3G), indications of typical metaplastic breast carcinomas undergoing EMT. A *p18*$^{-/-}$;*Brca1*$^{f/f}$;MC tumor expressed less than 10% *Brca1* and *Gata3* when compared to tumor-free mammary of the same mouse (Fig. 3I). FACS showed that the LSC-enriched population in *p18*$^{-/-}$;*Brca1*$^{f/f}$;MC mammary tumors was significantly reduced in comparison to the tumor-free mammary tissues of the same mouse (6% versus 56%) and when compared to *p18*$^{-/-}$ mammary tumor cells (6% versus 57%). Contrastingly, the BSC-enriched population, also enriched with breast CSCs, was significantly expanded in *p18*$^{-/-}$;*Brca1*$^{f/f}$;MC mammary tumors relative to the tumor-free mammary tissues of the same mouse (19% versus 11%) and when compared to *p18*$^{-/-}$ mammary tumor cells (19% versus 4%) (Fig. 3H). These results further support that *p18*$^{-/-}$;*Brca1*$^{f/f}$;MC mammary tumors are basal-like tumors undergoing EMT that are enriched with CSCs, which is in line with the data derived from human patients showing that metaplastic breast carcinomas are basal-like breast cancers with EMT-like molecular make-up and are closely correlated with BRCA1 dysfunction(18).

Taken together, these results suggest that insufficient Brca1 in mammary epithelial cells represses Gata3, activates Twist and EMT, and results in basal-like tumorigenesis with an increase in the CSC population. Since *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC and *p18*$^{-/-}$;*Brca1*$^{f/f}$;MC mice are in B6 and Balb/c mixed backgrounds, unlike *p18*$^{-/-}$;*Brca1*$^{+/-}$ mice in pure Balb/c background, these data also suggest that the role of Brca1 controlling basal-like tumorigenesis and EMT is independent of genetic background.

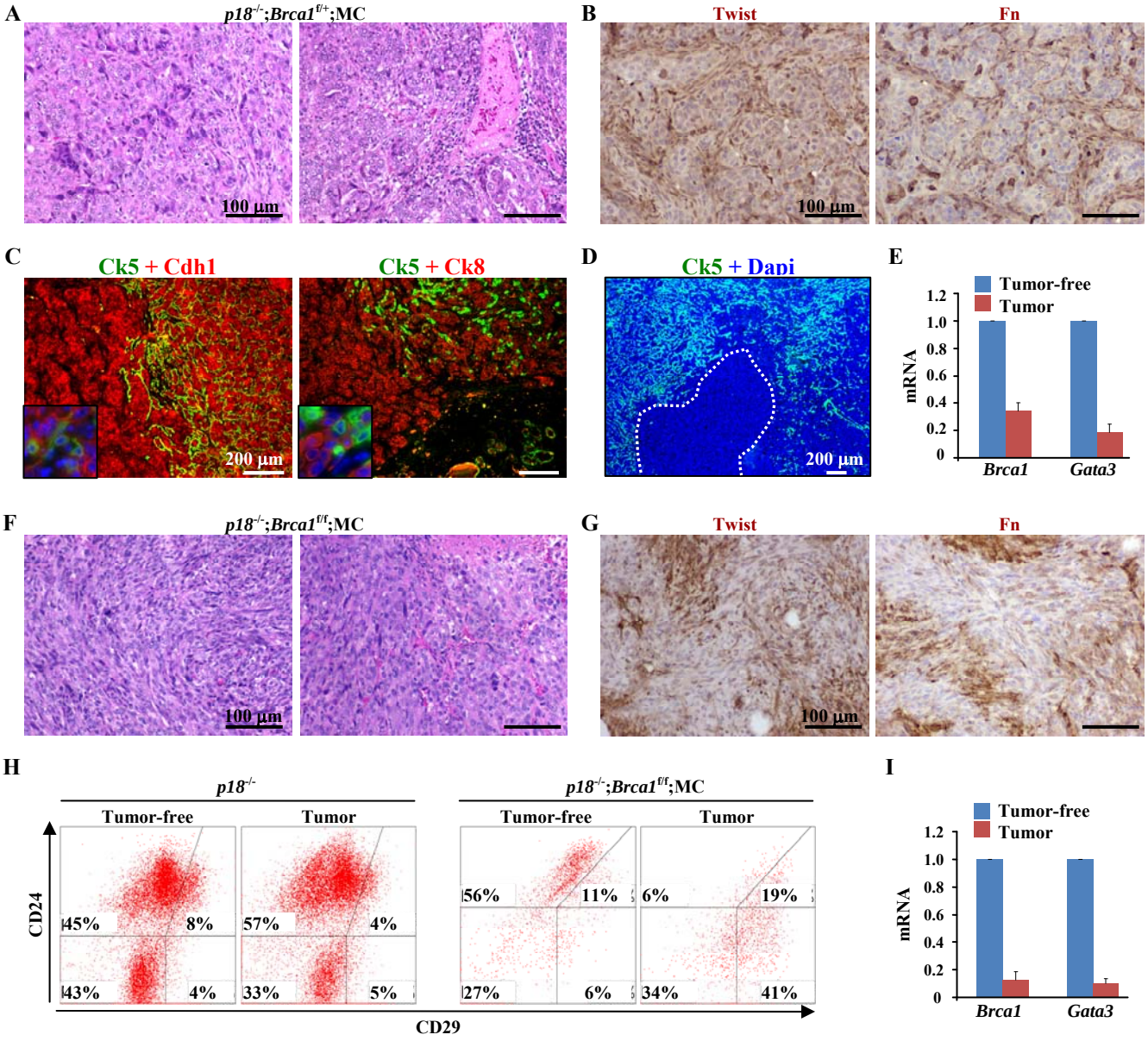

Figure 3. Deletion of Brca1 in mammary epithelia recapitulates basal-like tumor formation and EMT activation. Mammary tumors derived from *p18*$^{-/-}$;*Brca1*$^{f/+}$;MC (A-E) or *p18*$^{-/-}$;*Brca1*$^{f/f}$;MC (F-I) mice were stained with H.E (A, F), Twist or Fn (B, G), Ck5 and Cdh1 or Ck8 (C), Ck5 (D), or analyzed by Q-RT-PCR (E, I) and FACS (H). Tumor-free mammary cells or tissues from the same mouse were used as controls. Q-RT-PCR data are expressed as the mean ± SD from triplicates.

(4) BRCA1 suppresses TWIST transcription and EMT

We screened a panel of human breast cancer cell lines and found that MCF7 and T47D cells expressed higher CDH1 and GATA3 and lower VIM and EMT-TFs than SUM149 and HCC1937 cells (data not shown), confirming that MCF7 and T47D cells are luminal/epithelial-like and SUM149 and HCC1937 cells are basal/mesenchymal-like cancer cells in our culture system(19). Transfection of WT *BRCA1* into HCC1937 (*BRCA1* mutant, transcriptionally null) cells resulted in increase of *CDH1* and decrease of *VIM* and *FN*, indicating that BRCA1 suppresses EMT. Importantly, ectopic expression of BRCA1 significantly repressed

TWIST by more than 50% compared to control, moderately repressed *FOXC2*, but hardly repressed other EMT-TFs (Fig. 4A). A similar inhibitory effect on *TWIST* and *FOXC2* expression was also detected in 293T cells transfected with BRCA1 (data not shown). Since the ability of BRCA1 in regulating transcription controls normal differentiation and suppresses tumor development(20-21), we determined whether BRCA1 is recruited to the *TWIST* promoter. A previous study demonstrated that GATA3 recruits BRCA1 to its binding sites in the *FOXC1/2* promoters to repress their transcription(22). We performed bioinformatic analysis of the *TWIST* gene promoter and found that there exists, at the least, six putative GATA3 binding sites on the *TWIST* promoter (Fig. 4B), which are conserved in both human and mouse (data not shown). We then performed a ChIP assay and found that one of five amplicons that contained two GATA3 sites was specifically enriched in the immunoprecipitation of BRCA1 in HCC1937 cells transfected with WT *BRCA1* compared to control (P5 in Fig. 4C). In sum, these results suggest that BRCA1 specifically binds to the TWIST promoter and negatively regulates its transcription.

To confirm the role of Brca1 in the suppression of Twist and tumor development *in situ*, primary mammary tumors derived from $p18^{-/-}$;$Brca1^{+/-}$ mice were immunostained with antibodies against Brca1 and Twist. We found that tumor cells positive for Brca1 expressed very low or no Twist whereas Brca1 mutant tumor cells expressed high levels of Twist, most of which were spindle-shaped basal-like cells (Fig. 4D), demonstrating that Brca1 inhibits Twist and EMT in mammary tumor development and progression.

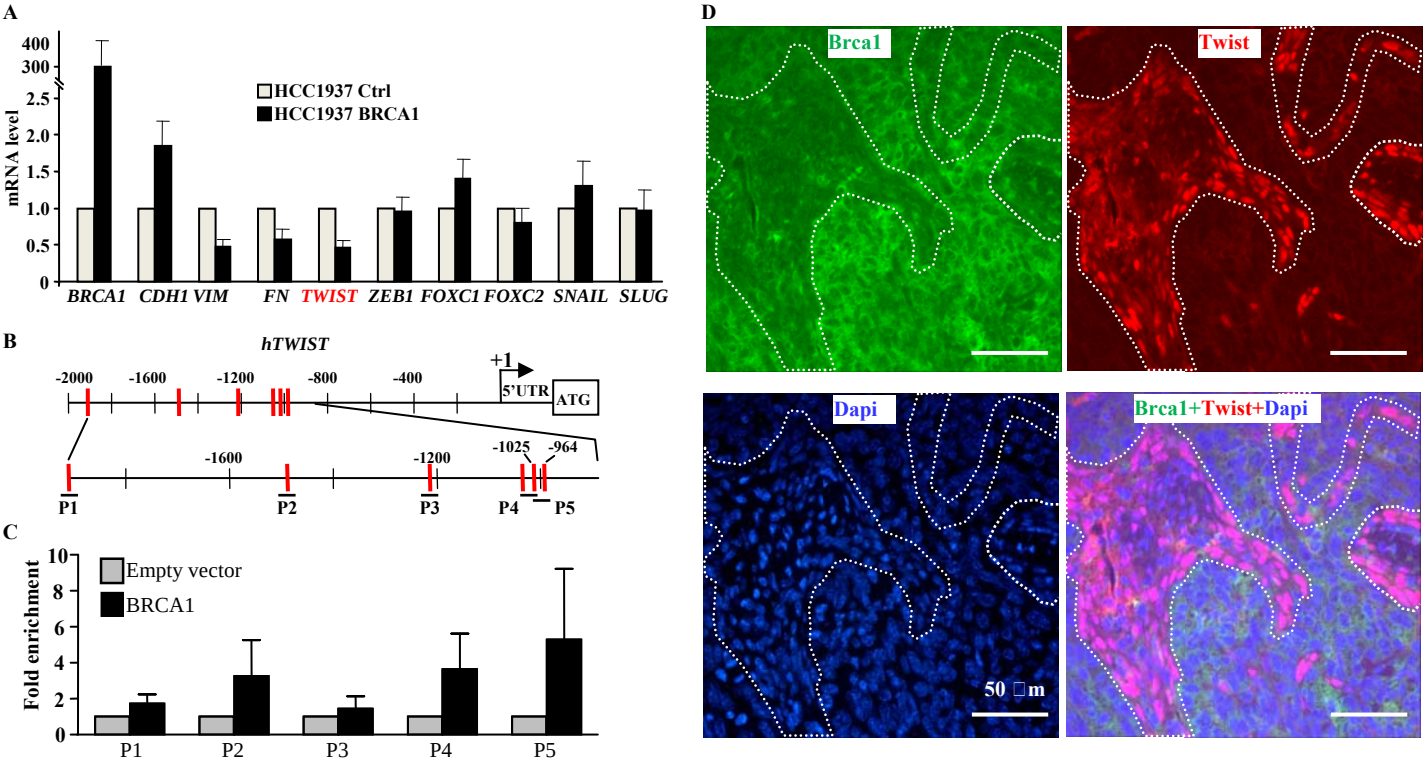

Figure 4. BRCA1 suppresses TWIST and EMT in mammary tumor cells. (A) HCC1937 cells were transfected with pcDNA3-empty (Ctrl) or pcDNA3-BRCA1 (BRCA1) and RNA was analyzed. Data are expressed as the mean ± SD from triplicates of two independent experiments. (B) Diagram showing the locations of putative GATA3 sites in the human *TWIST* gene. (C) ChIP analysis of BRCA1 binding to putative GATA3 sites on the *TWIST* promoter in HCC1937 cells transfected with *BRCA1*. Data are expressed as the mean ± SD from triplicates of two independent experiments. (D) Mammary tumors from $p18^{-/-}$;$Brca1^{+/-}$ mice were stained with antibodies against Brca1 (green) and Twist (red).

4. Key Research Accomplishments

Key accomplishments during the first year of the study were:

(1) Discovery that disrupting *Brca1* by either germline or epithelium-specific mutation in p18-deficient mice activates EMT and induces dedifferentiation of luminal stem cells, which associate closely with expansion of basal and cancer stem cells and formation of basal-like tumors.

(2) Discovery that BRCA1 binds to the *TWIST* promoter, suppressing its activity and inhibiting EMT in mammary tumor cells.

5. Conclusion

Our findings showed that BRCA1 suppressed TWIST and EMT, inhibited LSC dedifferentiation and repressed expansion of basal stem cells and basal-like tumors. Thus, our work offers the first genetic evidence that Brca1 directly suppresses EMT and LSC de-differentiation during breast tumorigenesis

6. Publications, Abstracts, and Presentations

Meeting Presentation:

Bai F, Chan HL, Scott A, Perou CM, and **Pei XH**. BRCA1 suppresses epithelial-to-mesenchymal transition in basal-like tumorigenesis. An AACR special conference-Advances in breast cancer research. October 3-6, 2013. San Diego, CA.

7. Inventions, Patents, and Licenses

Nothing to report

8. Reportable Outcomes

We found that disrupting *Brca1* in p18-deficient mice activates EMT and induces dedifferentiation of LSCs, which associate closely with expansion of basal and cancer stem cells and formation of basal-like tumors. Mechanistically, BRCA1 bound to the *TWIST* promoter, suppressing its activity and inhibiting EMT in mammary tumor cells. Our findings showed that BRCA1 suppressed TWIST and EMT, inhibited LSC dedifferentiation and repressed expansion of basal stem cells and basal-like tumors. Thus, our work offers the first genetic evidence that Brca1 directly suppresses EMT and LSC de-differentiation during breast tumorigenesis.

9. Other Achievements

Generation of p18;Brca1 double mutant mouse strains that develop basal-like breast cancers with high penetrance.

10. References

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11. Appendices

Nothing to report